Reference: *Biol. Bull.*, 139: 138–150. (August, 1970)

THE EVALUATION OF THE "*CALLIPHORA* TEST" AS AN ASSAY FOR ECDYSONE[1]

G. FRAENKEL AND JAN ZDAREK[2]

Department of Entomology, University of Illinois, Urbana, Illinois 61801

Thirty-five years ago one of us (Fraenkel, 1935) showed that abdomens of a fly larva, *Calliphora erythrocephala,* which had been isolated by ligation before a critical period and normally would never pupariate, could be induced to tan by injection of hemolymph from pupariating larvae. This experiment was confirmed on *Calliphora* (Becker and Plagge, 1939) and *Didacus ciliatus* (Narayanan and Lai, 1954). It subsequently became the method, now commonly known as the "*Calliphora* test," by which ecdysone was isolated, and with little modification is still the only practical method for identifying and assaying ecdysone. The validity of the original experiment was recently cast in doubt by Ohtaki, Milkman and Williams (1968) who failed to obtain pupariation by the injection of hemolymph in a member of a different family, *Sarcophaga peregrina.* They also used for their assays animals prepared in an entirely different manner, and on the basis of their data considered the 1935 experiment an erroneous observation, based on the appearance of "false positives." They stated that the entire larva at the critical period contained only 7% of a *Sarcophaga* unit of ecdysone, and quoted estimates of the ecdysone titre in *C. erythrocephala* by Shaaya and Karlson (1965) in support of this contention. A more recent analysis of the distribution of ecdysone in different tissues of *C. erythrocephala* by Shaaya (1969) was also in agreement with these views.

We have now reproduced the original results in *C. erythrocephala* and a related species, *Phormia regina,* as well as in *Sarcophaga argyrostoma.* Further probing into the conditions under which tanning occurs during puparium formation of flies led to the discovery of a neurohormonal effect which accelerates the onset of tanning (Zdarek and Fraenkel, 1969). This observation suggested that under natural conditions, the amount of ecdysone required for tanning might be smaller than what had hitherto been determined by the standard test as a *Calliphora* (or *Musca* or *Sarcophaga*) unit.

These considerations further led to an analysis of the various factors which affect the outcome of the traditional *Calliphora* test in four different species of flies: *Calliphora erythrocephala, Phormia regina, Sarcophaga bullata,* and *S. argyrostoma.*

Some preliminary remarks about the terminology concerning the *Calliphora* test for ecdysone are in place. Not only has the test been, and will be increasingly used with other fly species, such as *S. peregrina* (Oktaki, Milkman and Williams, 1967), *S. bullata, S. argyrostoma,* and *P. regina* (this paper), *Musca domestica*

[1] This study was supported by NSF grant GB 5441X and NIH grants AI-00533 and 5-K06-GM-18,495.

[2] On leave from the Institute of Entomology, Czechoslovak Academy of Science, Prague.

(Kaplanis, Tabor, Thompson, Robbins and Shortino, 1966; Adelung and Karlson, 1969), but several steroids of the general structure of ecdysone have been employed. We shall refer to the test as the "pupariation test for ecdysone," speak of ecdysone(s) when referring to the whole group of compounds, and use the terms α-ecdysone and β-ecdysone (rather than the synonyma ecdysterone, crustecdysone, 20-hydroxyecdysone) when referring to a particular compound. Instead of using "*Calliphora* unit" as a general term we shall refer to the particular fly species to which a particular determination applies, or use the general term "pupariation unit of ecdysone."

Materials and Methods

Species of flies used

Specimens of *Calliphora erythrocephala* Meigen, and *Phormia regina* L. (both Calliphoridae), *Sarcophaga bullata* Parker and *S. argyrostoma* Robineau-Desvoidy (Sarcophagidae) were used.

Breeding techniques

In much of the work reported below we used a modification of the culture technique which was suggested to us by Dr. W. L. Downes, Michigan State University. The feeding fly larvae with their food (pork liver) are loosely wrapped in a sheet of aluminum foil and placed on top of a layer of sawdust. Larvae which have finished feeding leave the food and descend into the sawdust. By removing larvae from the sawdust at regular intervals, groups of larvae are obtained of much more homogeneous physiological age than when keeping the whole batch together.

Ligation

In the standard tests larvae were ligated at a time when about 10–20% had already pupariated. Ligatures, using cotton thread, were placed well behind the ganglionic mass, approximately behind the 5th visible segment, at a distance of about one-third of the length of the larva counting from the front.

Injection

Hind sections of ligated larvae were injected by means of finely drawn glass pipettes with a diameter of about 40 μ at the tip, manufactured with the aid of a Micropipette Puller MI (Industrial Science Associates, Inc., Ridgewood, New York). Pressure was applied by mouth via a piece of rubber tubing fixed to the pipette. Amounts injected were estimated by comparison with a calibrated length of the same tubing. Since the hind parts of larvae ligated in this position are paralyzed, no further immobilization is required. Solutions of ecdysone in distilled water were injected in volumes of 5 μl or less per test abdomen.

Scoring

The degree of tanning produced in response to a particular injection was estimated by a method similar to that developed by the Karlson group (Karlson and

Shaaya, 1964; Adelung and Karlson, 1969) and others. These authors score complete, marked, half, and no pupariation as 100%, 75%, 50% and 0%, with a 25% score added in the latest paper. We use the same principle, with the difference, that all partial pupariation was scored as 50%. Our procedure yields essentially the same percentage figures of pupariation as that obtained by other authors.

The ecdysone used throughout was β-ecdysone (obtained from Syntex Corp., Palo Alto, California). The reason for using β- rather than α-ecdysone were: (1) Its availability. (2) Demonstration that this form is the one present in fly larvae (Galbraith, Horn, Thompson, Neufeld and Hackney, 1969). (3) Demonstration that β-ecdysone is more active in *Sarcophaga peregrina* than α-ecdysone (Ohtaki, Milkman and Williams, 1967).

Experiments

I. *The effect of ligation on pupariation in the front and hind parts of the larvae of four different fly species*

When a mature fly larva, with the crop empty, is ligated into two parts behind its ganglionic mass the anterior or posterior parts may or may not pupariate. It is generally known that if the ligature is made before a "critical period" only the anterior parts pupariate whereas the posterior parts remain untanned unless supplied with molting hormone (ecdysone). If ligated after the critical period, both sections tan, though the anterior part does so about two hours before the posterior part (Zdarek and Fraenkel, 1969). One would expect the anterior part to pupariate under all circumstances, since it contains the ring gland, the source of ecdysone. This is, however, not the case. The ligature seems to affect pupariation in the front part in various ways. Even when ligated after the critical period (when the hind part tans) the anterior part may fail to pupariate (for reasons never satisfactorily explained). This we consider an inhibition. We must expect similar incidents of inhibition also to occur in precritically ligated larvae. Ligation can also cause a delay in pupariation of the front part.

The foregoing remarks apply to all cyclorrhaphous flies, there are however, as will be presently shown, important specific differences in detail which have to be considered when drawing conclusions from one species to another. To this end we have timed the occurrence of pupariation in the front and hind parts of *C. erythrocephala, P. regina, Sarcophaga bullata* and *S. argyrostoma* after ligation and in relation to pupariation of unligated controls. The larvae were ligated when about 10% had already pupariated.

The reason why we have selected the 48 hours mark for discussing the results, and why the observations were not continued for much longer was the growing incidence of mortality after this period, and the increasing difficulty of discerning between anterior parts which are either dead, dying, or else no longer able to pupariate. The results are recorded in Figure 1, A–D.

In all four species the hind parts which pupariate do so about two hours after the front parts. (Cases where the posterior parts alone pupariated are excluded). No pupariation occurred in the hind parts later than 15 hours after ligaturing in *Calliphora* and *Phormia,* and later than 20 hours in the *Sarcophaga* species. The occurrence of "false positives" after a waiting period of 24 hours has not been

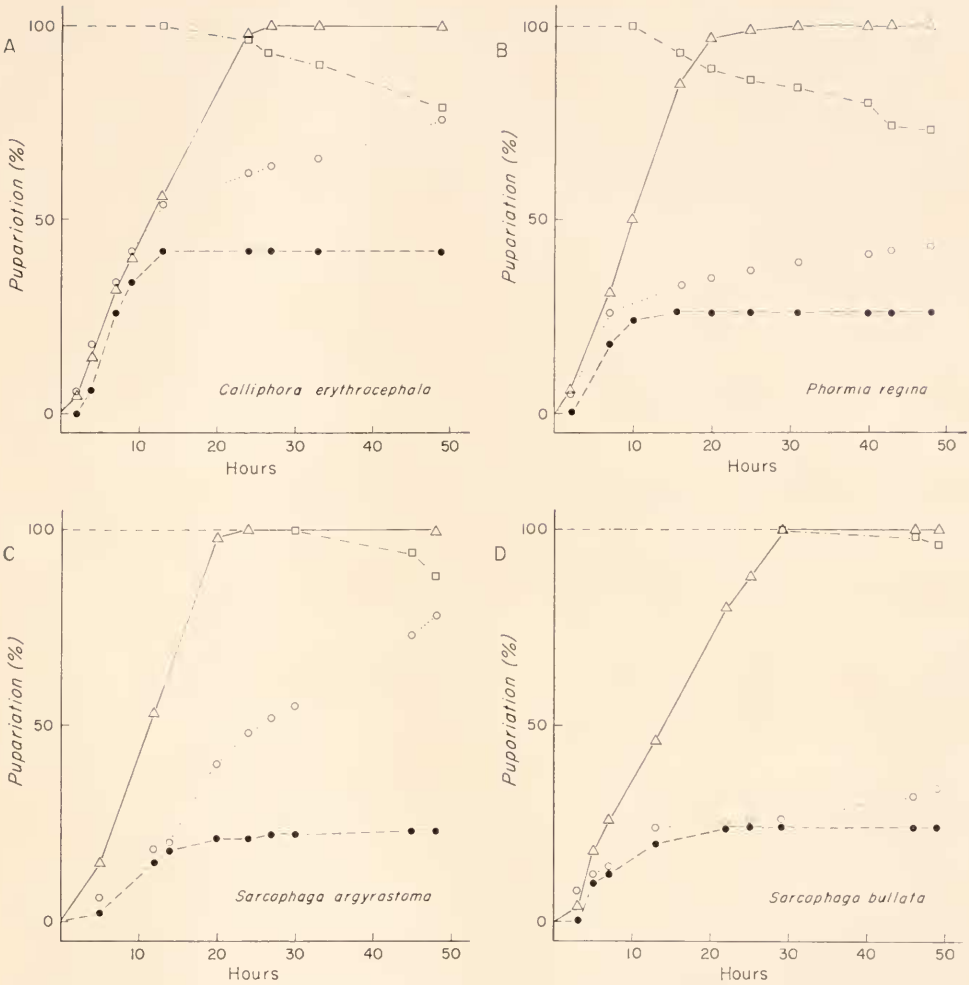

FIGURE 1. These graphs depict the course of pupariation in four species of flies in unligated controls △——△, the front parts ○·····○ and hind parts ●---● of ligated mature larvae. The top line □-·-·-□ represents mortality. A. *Calliphora erythrocephala.* B. *Phormia regina.* C. *Sarcophaga argyrostoma.* D. *Sarcophaga bullata.*

recorded. This eliminates the need for a further waiting period. Pupariation in the anterior parts in relation to that in the non-ligated controls does not seem to be delayed to any significant degree during the first 5 to 10 hours after ligating, but such a delay becomes very considerable in precritically ligated specimens, and is more marked in *P. regina* and *S. bullata,* than *C. erythrocephala* and *S. argyrostoma.*

In addition to this delay there is also an inhibition whereby many anterior parts fail to pupariate altogether. The incidence of this inhibition was very different in the different species. The proportion of precritically ligated larvae which by

48 hours after ligating had puparіated in the anterior part was 12% in *S. bullata,* 23% in *P. regina,* 59% in *C. erythrocephala* and 71% in *Sarcophaga argyrostoma.* The total percentage of larvae which had not puparіated in the front part after 48 hours was 65% for *S. bullata,* 57% for *P. regina,* 23% for *C. erythrocephala,* and 22% for *S. argyrostoma.* These figures are not the final results because more puparіation occurred at a later date. The count was terminated at this date for reasons stated above. Despite this uncertainty, the different trend in the different species is very clear.

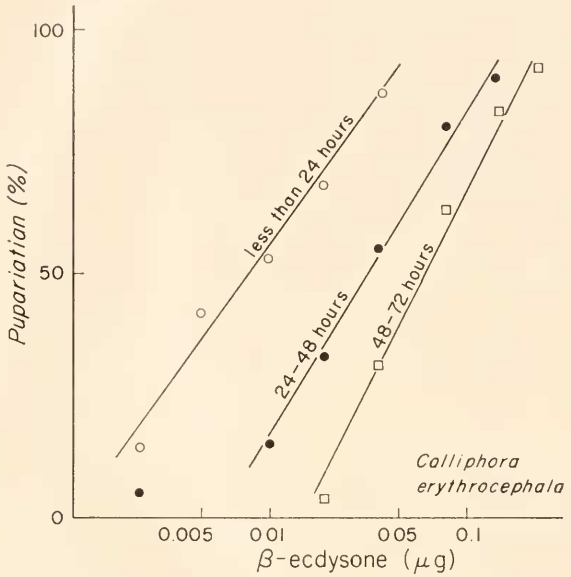

FIGURE 2. *Calliphora erythrocephala.* The effect of the injection of graded doses of β-ecdysone on puparіation in test abdomens from larvae where the anterior part alone had puparіated within 24 hours, between 24 and 48 hours, and between 48 and 72 hours after ligation.

It is interesting that in *S. bullata* and *P. regina,* where the delaying and inhibitory effect on puparіation in the precritically ligated larvae is stronger than in *C. erythrocephala* and *S. argyrostoma,* ligation after the critical period gave rise to many more specimens where the hind part alone puparіated (*S. bullata* 16%, *P. regina,* 45%, *S. argyrostoma* 4%, and *C. erythrocephala* 3%).

Since the first prerequisite for a successful ecdysone test is as high a yield as possible of specimens which puparіate in the anterior part alone within the first 24 hours after ligating, *S. bullata* and *P. regina* are far less suitable test subjects than *C. erythrocephala* and *S. argyrostoma.*

II. *Variability of the puparіation unit of ecdysone*

Karlson and Hanser (1953), Karlson and Shaaya (1964), and Adelung and Karlson (1969) have previously shown that the amount of ecdysone necessary to induce puparіation in the ligatured hind part is very much dependent on the

physiological age of the preparation at the time of injection. This age effect should be considered from two different perspectives. One is the presumed age of the larva in relation to pupariation which, in view of the large individual variation in any one batch can only be guessed from the time of pupariation in the controls, and from the period between ligation and pupariation in the front part. Since, as seen in Figure 1, pupariation in the normal controls was about completed after 24 hours, we may assume that all anterior parts which are tanned by that time had done so at more or less their appropriate time. All pupariation subsequently is increasingly delayed. The second consideration concerns the time interval between pupariation in the front part and the actual injection.

TABLE I

Calliphora erythrocephala, *effect of delay of injection of ecdysone on pupariation of test abdomens. β-Ecdysone was injected at different periods into test abdomens of larvae in which the anterior part had pupariated within 24 hours after ligaturing. Each horizontal line represents tests performed simultaneously on larvae of the same batch, n = number of test abdomens per test, % = score of pupariation.*

β-Ecdysone per test abdomen (μg)	Score of pupariation in test abdomens injected at the following periods after ligaturing					
	24 hours		48 hours		72 hours	
	n	%	n	%	n	%
0.006	12	42	11	32		
	12	41	10	30		
0.012	11	82	11	64		
	11	45	10	40		
0.025	11	86	11	64		
	12	54	10	50		
0.05	9	94	8	87		
0.1	11	82	12	63	9	50
0.2	11	95	12	87	10	60

1. *Injection into test abdomens of different physiological ages.* Mature larvae of *Calliphora erythrocephala* were ligated, and the larvae which subsequently pupariated in only the front section divided into three groups according to the interval between ligation and pupariation: (1) Within 24 hours; (2) Between 24 and 48 hours; (3) Between 48 and 72 hours after ligation. Group 1 was injected 24 hours, group 2, 48 hours, group 3, 72 hours after ligation. Responses to injection of each particular dose of ecdysone were estimated on the basis of three assays in group 1 and 2, and 2 assays in group 3, comprising 10 to 15 specimens at each assay. It was necessary to make several replications for each dose to draw the straight lines of Figure 2, since response to a particular dose varied considerably when larvae from different batches were used. This, by itself, shows that the bioassay is not very well reproducible.

The results (Fig. 2) show that test abdomens of group 1 were greatly more sensitive than of group 2, with those of group 3 still less sensitive. The value of the *Calliphora* unit, as defined above, was 0.008, 0.035, and 0.07 μg β-ecdysone per test abdomen for groups 1, 2, and 3, respectively.

2. *Injection into test abdomens of the same physiological age after different waiting periods.* The posterior parts of larvae whose anterior part alone had puparíated up to 24 hours after ligating were injected with graded doses of β-ecdysone at 24, 48, and 72 hours after ligating. The result (Table I) shows a lower sensitivity of test abdomens to injected β-ecdysone when the test abdomens had been further "aged." The differences between the 24 and 48 hours injections are significant at the 0.004 level (two tailed), by the Wilcoxon paired-sample rank test.

3. *The dilution effect.* In the puparíation test for ecdysone various authors injected the hormone solutions in portions of 10 (*Calliphora, Sarcophaga*) or 5 μl (*Musca*) (see Discussion). This amount of solvent represents a far from negligible addition to the blood volume of the test abdomens. In order to examine the effect of such a dilution on puparíation β-ecdysone was injected into test abdomens of *C. erythrocephala* in volumes of 1 and 10 μl water. Table II shows that the puparíation score was consistently reduced with the higher volumes of injection.

TABLE II

Calliphora erythrocephala, *effect of the volume of injection on the puparíation score of test abdomens injected with ecdysone. Injections were made 24 hours or 48 hours after ligation (the anterior parts had puparíated before 24 hours or between 24 and 48 hours after ligation, respectively).*

"Age" of test abdomens hrs	β-Ecdysone (μg) per test abdomen	Score of puparíation			
		Injected in 1 μl		Injected in 10 μl	
		n	%	n	%
24	0.01	15	60	15	40
24	0.01	12	58	12	33
48	0.02	12	37	12	18

4. *The effect of a brain hormone on the puparíation unit of ecdysone.* In a preceding paper (Zdarek and Fraenkel, 1969) we had shown that the addition of an extract from neurosecretory cells in the brain not only accelerated the onset of puparíation but also increased the sensitivity of the test abdomens to injected ecdysone in *Phormia regina.* We have now investigated this phenomenon in greater detail in *C. erythrocephala.*

Precritically ligated larvae were divided into three groups according to the time when the anterior parts alone had puparíated: A. within 24 hours, B. between 24 and 48 hours, C. between 48 and 72 hours after ligation. The hind parts were injected with graded doses of β-ecdysone dissolved in water, and with a homogenate of CNS of *S. bullata* larvae from which the ring glands had previously been removed (Table III). Test abdomens of group A required less ecdysone for a given puparíation effect when also injected with the CNS extract. In applying the Wilcoxon paired-sample rank test, the percentages given in Table 3A were recalculated to the first place of decimals to resolve the ties between the first and fifth experiment. The differences in puparíation score in the presence or absence of CNS extracts were significant at the 0.04 level, two-tailed. Puparíation

TABLE III

Calliphora erythrocephala, *effect of graded dose of β-ecdysone on pupariation of test abdomens ligated at different physiological ages, in the presence or absence of homogenates from brains of* Sarcophaga bullata.

Injected per test abdomen		Pupariation score in absence of brain homogenate		Pupariation score in presence of brain hormone			Difference of percentage
vol. μl	β-ecdysone μg	n	%	brain equivalent*	n	%	
A. Anterior parts pupariated within 24 hours after ligation; injected 24 hours after ligation.							
5	0.004	10	35	1.5	13	27	−8
5	0.008	17	32	1.5	16	47	+15
10	0.01	9	17	2.0	10	55	+38
5	0.016	16	59	1.5	16	66	+7
10	0.02	14	46	2.0	14	54	+8
5	0.032	16	81	1.5	16	91	+10
10	0.04	14	61	2.0	14	79	+18
B. Anterior parts pupariated 24 to 48 hours after ligation; injected 48 hours after ligation.							
5	0.004	11	27	1.5	17	6	−21
5	0.008	12	29	1.5	17	3	−26
5	0.016	11	54	1.5	17	9	−45
5	0.032	16	84	1.5	15	57	−27
10	0.05	12	21	2.0	11	45	+24
10	0.1	12	58	2.0	11	72	+14
7	0.05	12	58	1.5	12	42	−16
7	0.1	12	67	1.5	12	71	+4
7	0.2	12	100	1.5	12	75	−25
C. Anterior parts pupariated 48 to 72 hours after ligation; injected 72 hours after ligation.							
10	0.1	11	36	2.0	11	18	−18
10	0.2	11	68	2.0	11	32	−36

* Number of brains per abdomen.

was also considerably accelerated in the presence of the CNS extracts. The larvae of group B and C which had been ligated at a younger age did not show either of these effects. Those of group C seemed to show an opposite effect of decrease of ecdysone action in the presence of the neurohormone.

III. *Pupariation induced in test abdomens of different fly species by the injection of hemolymph*

In order to test abdomens at their most sensitive state (see above) most injections were made 24 hours or earlier after ligaturing. Twenty-four hours after the injection the number and degree of pupariation, as well as the number of dead specimens, were recorded. The surviving untanned specimens which ap-

peared in a sufficiently healthy state were then injected a second time and again scored after 24 hours.

The results are shown in Table IV. The final column, giving the total ratio, sums up the results of the first and second injection with the percentages calculated from the number of survivors after the first injection.

TABLE IV

Puparium formation in test abdomens of larvae of Calliphora erythrocephala, Phormia regina, Sarcophaga bullata *and* S. argyrostoma *as a result of injecting them with hemolymph from different donors at different times after the critical period.*

Test abdomens, species, time of injection after ligation	Hemolymph injected		Pupariation 24 hrs after				Total pupariation score	
	amount test abdomen	Donor	1st injection		2nd injection*			
			n	%	n	%	n	%
C. erythrocephala								
24 hrs	10 µl	*C. erythr.*, white puparia	26	35	14	11	26	40
24 hrs	10 µl	*C. erythr.*, feeding larvae	38	3	32	6	38	8
24 hrs	10 µl	*S. bullata*, white puparia	17	0	8	0	17	0
24 hrs	10 µl	*S. argyr.*, white puparia	15	37	5	20	15	43
48 hrs	10 µl	*C. erythr.*, white puparia	35	0	27	20	35	16
48 hrs	10 µl	*C. erythr.*, feeding larvae	27	0	22	0	27	0
48 hrs	10 µl	*S. bullata*, white puparia	25	0	22	0	25	0
P. regina								
16 hrs	5 µl	*P. regina*, white puparia	24	12	16	16	24	23
16 hrs	5 µl	*P. regina*, feeding larvae	15	0	14	0	15	0
12 hrs	8–10 µl	*P. regina*, white puparia	23	22	16	25	23	39
12 hrs	8–10 µl	Ringer sol.	10	10	9	0	10	10
S. bullata								
24 hrs	10–15 µl	*S. bullata*, white puparia	30	2	26	2	30	3
24 hrs	10–15 µl	*S. bullata*, feeding larvae	24	6	19	0	24	6
24 hrs	15 µl	*C. erythr.*, white puparia	23	4	22	2	23	7
24 hrs	15 µl	*C. erythr.*, feeding larvae	22	0	22	0	22	0
S. argystoma								
24 hrs	15 µl	*S. argyr.*, white puparia	32	2	31	2	32	3
24 hrs	25 µl	*S. argyr.*, white puparia	16	28	11	14	16	37
24 hrs	15 µl	*C. erythr.*, white puparia	15	0	—	—	—	—
24 hrs	25 µl	*C. erythr.*, white puparia	20	40	9	22	20	45
24 hrs	10 µl	*S. argyr.*, concentrate**	15	17	—	—	—	—
24 hrs	25 µl	*S. argyr.*, concentrate**	12	67	—	—	—	—
24 hrs	25 µl	Ringer sol.	18	6	—	—	—	—
24 hrs	—	nothing injected	26	4	—	—	—	—

*Second injection into surviving larvae, 24 hours after first.

** Filtrate from heat-coagulated blood from white puparia concentrated 5 times.

There were conspicuous differences between the four species. *Calliphora, Phormia,* and *S. argyrostoma* yielded pupariation scores around 40%, figures similar to those previously reported, but only when tested under the most favorable circumstances. It was essential to control the time and dosage of injection. *Calliphora* responded when injected 24 hours, but not 48 hours after ligation. *S. argyrostoma* responded well to injection of 25 µl of its own hemolymph, but not

at all to that of 15 μl. It responded equally well to 25 μl but not at all to 15 μl of *Calliphora* hemolymph. Reversely, *Calliphora* responded to 10 μl of hemolymph from *S. argyrostoma,* but not to that from *S. bullata.* This would suggest that the activity of the hemolymph of *S. argyrostoma* is higher than that of *S. bullata. S. bullata* showed no response to 10–15 μl of its own hemolymph or that of *Calliphora.* No further tests were undertaken with this species in view of the difficulty of obtaining suitable test abdomens (see above).

Active hemolymph was taken throughout from white puparia which, according to Shaaya and Karlson (1965) and Shaaya (1969), are at the peak of ecdysone activity. Hemolymph from younger, still feeding larvae (*i.e.,* at least a day before the critical period) was in all cases inactive, thus proving the specificity of the test.

In view of the larger size of *Sarcophaga* it is not surprising that a larger volume of injected hemolymph is required for pupariation. An attempt was made of preparing concentrates of hemolymph for the purpose of increasing its ecdysone content. Hemolymph was diluted with 3 parts of water, heated at 90° for 10 minutes, centrifuged, and the supernatent concentrated on the vacuum evaporator at 70° C to one-fifth of the original concentration. Ten microliters of this concentrate evoked a weak but clear tanning response, while 25 μl led to 67% pupariation. A comparison of the effects of 25 μl of the original blood and 10 μl of the concentrates shows that some activity was lost in the course of preparation of the concentrate.

Discussion

It has recently been several times reported that the ecdysone titre in the hemolymph of larvae of cyclorrhaphous flies prior and during pupariation is far lower than the amount of exogenous hormone necessary to cause pupariation in the pupariation test for ecdysone (Shaaya and Karlson, 1965; Ohtaki, Milkman and Williams, 1968; Shaaya, 1969). Yet, we (this paper) and others (Fraenkel, 1935; Becker and Plagge, 1939; Narayanan and Lai, 1954) have shown that the hemolymph of pupariating larvae is capable to induce tanning in the pre-critically ligated abdomens. In order to solve this paradoxical situation we analyzed the pupariation test in great detail and revealed a number of factors which can affect the outcome of the traditional test.

1. *Age of the larvae at the time of ligation*

We (Fig. 2), as similarly before Karlson and Hanser (1953), Karlson and Shaaya (1964), and Adelung and Karlson (1969), have shown that test abdomens become greatly more sensitive to ecdysone nearer the time of pupariation.

2. *Waiting period between ligation and injection*

The test abdomen loses in responsiveness if injection of ecdysone is delayed (Table I). In Ohtaki, Milkman and Williams (1968) standard test abdomens a waiting period of 40 hours increased the *Sarcophaga* unit from slightly over 0.01 to about 0.035 μg. Loss of sensitivity by post-ligation aging was also recorded by Karlson and Hanser (1953). Waiting periods of 24 hours or longer were routinely employed by all previous authors.

3. *A dilution effect*

When ecdysone is injected into test abdomens of *Calliphora* (Adelung and Karlson, 1969) or *Sarcophaga* (Ohtaki, Milkman and Williams, 1967) in portions of 10 μl of an aqueous solution, or dissolved in 10% isopropanol, respectively, or of 5 μl in *Musca* (Adelung and Karlson, 1969), the hemolymph of the receivers becomes appreciably diluted (the blood volume of a *Calliphora* test abdomen is 20 to 25 μl). The effect which this overall change in concentration (not only of ecdysone) may have on tanning does not seem to have been considered. It clearly leads to an overestimate of the pupariation unit of ecdysone (Table II). Such effects are also noticeable in the data of Table III.

4. *The effect of a neurohormone*

Our previous data (Zdarek and Fraenkel, 1969) from *P. regina* had suggested that a humoral factor from the brain which accelerated the onset of pupariation also increased the sensitivity of the test abdomens to injected ecdysone. The same effect has now been shown in *C. erythrocephala*, but only in relatively mature test abdomens (Table III). A similar "synergistic" effect of a combined action of a brain hormone and ecdysone, both prepared from *Bombyx*, in the pupariation of *C. erythrocephala* had been discovered by Kobayashi and Burdette (1961). Thus, the absence of the neurohormone, as is the case in the standard test for ecdysone, may increase the requirements for ecdysone.

In all our experiments where injection of hemolymph induced pupariation, test abdomens of the highest possible sensitivity were employed, *i.e.* ligated as closely as possible before the critical period, and injected without a further waiting period with the highest possible amount of hemolymph. Thus, in the light of the various factors enumerated above—age, dilution effect, presence of neurohormone—the true *Calliphora* unit of ecdysone under the most favorable conditions may be substantially lower than the figure now given for the synthetic hormone (0.01 μg/test abdomen). Furthermore, the hemolymph of pupariating larvae contains, besides the humoral factors, a potent substrate-enzyme system, which may not be without a significance for the result of the test. Our results with interspecific transfusion of hemolymph confirm not only the non-specificity of its activity (Becker and Plagge, 1939), but also suggest that this activity can vary in different species.

Ohtaki, Milkman and Williams (1968) in explanation of their data with *Sarcophaga peregrina* suggest a mechanism by which ecdysone acted by a gradual accumulation of covert effects in response to subthreshold doses, rather than by massive accumulation in the hemolymph. In a current investigation on *S. argyrostoma*, to be published shortly, we have obtained direct experimental evidence in support of this contention, namely, that the effects of both exogenous and endogenous ecdysone can and do summate over a period of time. In the light of these findings it may well be that the amount of hormone contained in injected hemolymph which induces tanning in test abdomens is only the last quantity necessary to bring about the qualitative change—tanning—in test abdomens already preconditioned (sensitized) by subthreshold doses of the hormone. Then the final effect is the results of two factors, sensitivity of the test abdomen and activity of the hemolymph.

In considering the significance of the pupariation test for ecdysone we must realize that the *Calliphora* (*Sarcophaga, Musca*) unit of ecdysone can only refer to the particular and peculiar conditions of the test abdomens, and may bear little relation to the real hormone titre in the normal larva at the time of pupariation which is also affected by the rates of inactivation (Ohtaki, Milkman and Williams, 1968; Karlson and Bode, 1969; Shaaya, 1969) and continuous release of ecdysone.

We thank our colleagues, Drs. J. Willis and G. Bhaskaran for reading critically the manuscript, Dr. A. Ghent for the statistical treatment, and Dr. J. B. Siddall of Syntex Research, Palo Alto, for the supply of β-ecdysone.

Summary

1. The effect of ligation on pupariation in the front or hind parts of larvae of four species of flies, *Calliphora erythrocephala, Phormia regina, Sarcophaga bullata*, and *S. argyrostoma* was investigated. Ligation causes effects of delay or inhibition of pupariation which are very differently expressed in the four species. A large proportion of pre- or postcritically ligated specimens of *P. regina* and *S. bullata* altogether fail to pupariate in the anterior part. This makes these species unsuitable test subjects for the pupariation test for ecdysone.

2. Test abdomens of *C. erythrocephala* required significantly less ecdysone for a given pupariation effect when also injected with a CNS-extract. Tanning was also considerably accelerated in this case.

3. The value of the pupariation unit of ecdysone is influenced by a number of factors, such as age at the time of ligation, the waiting period between ligation and injection, the dilution effect of the solvent, and the simultaneous action of a neurohormone. The requirements for natural ecdysone in normal larvae at the time of pupariation are probably substantially lower than the values which have been determined by others with test abdomens and the use of synthetic ecdysones.

4. In confirmation of older data, and contrary to recent claims, tanning was induced in test abdomens of the larvae of *C. erythrocephala, P. regina*, and *S. argyrostoma* by the injection of hemolymph from pupariating larvae. *Calliphora* blood induced tanning in specimens of *S. argyrostoma*, and vice versa. The conclusions are drawn that differences between the different species in the action of ecdysone are of a quantitative rather than qualitative nature.

LITERATURE CITED

Adelung, D., and Karlson, P., 1969. Eine verbesserte, sehr empfindliche Methode zur biologischen Auswertung des Insektenhormones Ecdyson. *J. Insect Physiol.*, **15**: 1301–1307.

Becker, E., and E. Plagge, 1939. Über das die Puppariumbildung auslösende Hormon der Fliegen. *Biol. Zbl.*, **59**: 326–341.

Fraenkel, G., 1935. A hormone causing pupation in the blowfly, *Calliphora erythrocephala*. *Proc. Roy. Soc. London, Series B*, **118**: 1–12.

Galbraith, M. N., D. H. S. Horn, J. A. Thompson, G. J. Neufeld and R. J. Hackney, 1969. Insect moulting hormones: Crustecdysone in *Calliphora*. *J. Insect Physiol.*, **15**: 1225–1233.

KAPLANIS, J. N., L. A. TABOR, M. I. THOMPSON, W. E. ROBBINS AND T. I. SHORTINO, 1966. Assay for ecdysone (moulting hormone) activity using the house fly, *Musca domestica* L. *Steroids*, **8**: 625–631.

KARLSON, P., AND C. BODE, 1969. Die Inaktivierung des Ecdysons bei der Schmeissfliege *Calliphora erythrocephala* Meigen. *J. Insect Physiol.*, **15**: 111–118.

KARLSON, P., AND G. HANSER, 1953. Bildungsort und Erfolgorgan des Puparisierungshormones der Fliegen. *Z. Naturforsch.*, **86**: 91–96.

KARLSON, P., AND S. SHAAYA, 1964. Der Ecdysontiter während der Insektenentwicklung. 1. Eine Methode zur Bestimmung des Ecdysonsgehaltes. *J. Insect Physiol.*, **110**: 797–804.

KOBAYASHI, M., AND W. J. BURDETTE, 1961. Effect of brain hormone from *Bombyx mori* on metamorphosis of *Calliphora erythrocephala*. *Proc. Soc. Exp. Biol. Med.*, **107**: 240–242.

NARAYANAN, E. S., AND R. LAI, 1954. Controlling mechanism of pupation in *Didacus ciliatus* (Loew.) (Diptera). *Indian J. Entom.*, **16**: 176–188.

OHTAKI, T., R. D. MILKMAN AND C. M. WILLIAMS, 1967. Ecdysone and ecdysone analogues: Their assay on the flesh fly *Sarcophaga peregrina*. *Proc. Nat. Acad. Sci.*, **58**: 981–984.

OHTAKI, T., R. D. MILKMAN AND C. M. WILLIAMS, 1968. Dynamics of ecdysone secretion and action in the flesh fly *Sarcophaga peregrina*. *Biol. Bull.*, **135**: 322–334.

SHAAYA, E., 1969. Der Ecdysontiter während der Insektenentwicklung, VI. Untersuchungen über die Verteilung in verschiedenen Geweben von *Calliphora erythrocephala* und über seine biologische Halbwertszeit. *Z. Naturforsch.*, **24b**: 718–721.

SHAAYA, E., AND P. KARLSON, 1965. Der Ecdysontiter während der Insektenentwicklung. II. Die postembryonale Entwicklung der Schmeissfliege *Calliphora erythrocephala* Meig. *J. Insect Physiol.*, **11**: 65–69.

ZDAREK, J., AND G. FRAENKEL, 1969. Correlated effects of ecdysone and neurosecretion in puparium formation (pupariation) of flies. *Proc. Nat. Acad. Sci.*, **64**: 565–572.